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Allelic discrimination by nick-translation PCR with fluorogenic probes

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C12Q1/68M6

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ABSTRACT

Nick-translation PCR was performed with fluorogenic probes. Two probes were used: one complementary to a sequence containing the F508 codon of the normal human cystic fibrosis (CF) gene (*wt* DNA) and one complementary to a sequence containing the ΔF508 three base pair deletion (*mut* DNA). Each probe contained a unique and spectrally resolvable fluorescent indicator dye at the 5′ end and a common quencher dye attached to the seventh nucleotide from the 5′ end. The F508/ΔF508 site was located between the indicator and quencher. The probes were added at the start of a PCR containing *mut* DNA, *wt* DNA or heterozygous DNA and were degraded during thermal cycling. Although both probes were degraded, each probe generated fluorescence from its indicator dye only when the sequence between the indicator and quencher dyes was perfectly complementary to target. The identity of the target DNA could be determined from the post-PCR fluorescence emission spectrum.

INTRODUCTION

Current methods of detecting PCR products rely on post-PCR separation of the desired PCR product from the reaction mixture and detection, based either on size (agarose or acrylamide gel electrophoresis) or sequence (probe-hybridization methods including the reverse dot blot and oligonucleotide ligation assays) (1). Automation of these methods is complicated by the heterogeneous nature of the assays. Holland *et al.* described a nick-translation assay in which the 5′→3′ exonuclease activity of *Taq* DNA polymerase cut a labeled probe during PCR (2). The 5′ ^{32}P-labeled probe was complementary to one strand of the PCR product. The probe was partially digested during the PCR by the 5′→3′ exonuclease activity of *Taq* DNA polymerase. Detection of the labeled fragments after separation from uncleaved probe permitted a determination of PCR specificity. The authors stated that with appropriate labeling, 'we envision the use of this technique to create a truly homogeneous assay'. Here, we demonstrate such a homogeneous system, in which no post-PCR separation is needed, with fluorogenic probes specific for the F508 and ΔF508 alleles of the human cystic fibrosis gene (3).

MATERIALS AND METHODS

Reagents

ATP-dependent DNase and BstNI-digested pBR322 DNA were purchased from United States Biochemical (Cleveland, OH). Amplitaq® DNA polymerase, 2′-deoxynucleotide 5′-triphosphates (dNTPs) and AmpliWax™ PCR Gems were purchased from Perkin-Elmer (Norwalk, CT). DNA was prepared and purified in a Model 341 Genepure DNA extractor (Applied Biosystems) from human cell lines purchased from Coriell Cell Repository (Camden, NJ). The cell lines were GM07824 (ΔF508 homozygote), GM07224 (F508 homozygote) and GM11274 (F508/ΔF508 heterozygote). DNA size standard BioMarker EXT was purchased from AT-Biochem (Malvern, PA).

Oligonucleotides

Linker arm nucleotide phosphoramidite (LAN) was purchased from Glen Research (Herndon, VA). 5′-Phosphate ON was purchased from Clontech (Palo Alto, CA). 6-carboxyfluorescein (6FAM) and 6-carboxy-4,7,2′,7′-tetrachlorofluorescein (TET) phosphoramidites, standard DNA phosphoramidites and 6-carboxytetramethylrhodamine, succinimidyl ester (TMR NHS ester) were obtained from Applied Biosystems. Oligonucleotide synthesis was performed using a Model 394 DNA synthesizer (Applied Biosystems). Doubly labeled probes were synthesized with a dye-labeled phosphoramidite at the 5′ end, LAN at the seventh position from the 5′ end, and 5′-phosphate ON at the 3′ end. Oligonucleotide purification was performed on a Perkin-Elmer Series 4 Liquid Chromatograph with an Applied Biosystems Aquapore RP300 column. Addition of the second dye to the LAN-tagged oligonucleotide was accomplished with TMR NHS ester, followed by Sephadex G50 purification to remove excess dye, and a second reverse-phase HPLC purification to remove unreacted oligonucleotide.

Probe digestion

A solution containing 0.5 μM fluorescent probe, 0.5 mM ATP, 20 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 80 mM Tris–HCl at pH 8.9 in a final volume of 0.4 mL was divided into two 0.2 mL portions. To one portion was added 13 U of ATP-dependent DNase. After overnight digestion at room temperature, the fluorescence spectra of both portions were measured.

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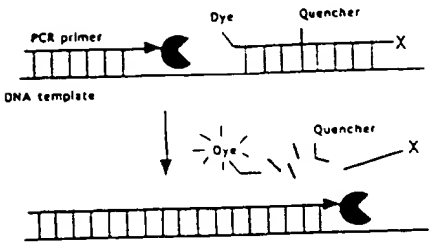

Figure 1. Use of fluorogenic probes in nick-translation PCR.

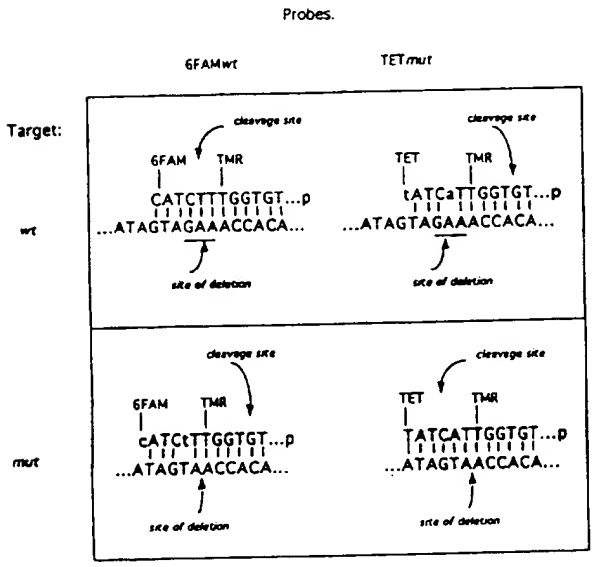

Figure 2. Discrimination between F508 and ΔF508 by two fluorogenic probes. Lower case letters represent mismatched nucleotides; p = 3'-phosphate. Top row: Two probes, 6FAM*wt* and TET*mut*, bound to *wt* DNA. Bottom row: The same two probes bound to *mut* DNA.

PCR

Hot Start amplifications (100 μL final volume) were done with AmpliWax™ PCR Gems in 0.5 mL Eppendorf tubes in a Perkin-Elmer/Cetus Thermal Cycler. Each tube contained 0.2 μM of one or both fluorescently labeled probes and PCR primers, 200 μM of each dNTP, 20 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 80 mM Tris−HCl at pH 8.9, and 5 U of DNA polymerase. Genomic DNA was added at $3-5\times10^4$ copies per reaction. Thermal cycling was performed with 1 min denaturation at 98°C for two initial cycles, followed by 35 cycles using denaturation at 95°C for 1 min. Annealing and extension were performed at 49°C for 1 min in each cycle.

Analysis of PCR products

Approximate size and yield of ds DNA PCR products were estimated by electrophoresis of 5 μL of each PCR reaction on an 0.8% agarose gel containing ethidium bromide. The bands were visualized by UV excitation.

Spectral and mobility characteristics of the degraded probes were determined by analysis of 0.3 μL of each PCR reaction in an Applied Biosystems Model 373 DNA sequencer with Model 672 analysis program. Electrophoresis was performed for 90 minutes at a constant power of 35 watts on a 6% denaturing polyacrylamide gel. The gel was examined using 10-nm bandpass filters centered at 531, 545, 560 and 580 nm (filter set B).

Fluorescence spectra of each PCR reaction were measured in 50 μL quartz cuvettes (Hellma Cells, Jamaica, NY) in a Perkin-Elmer LS-5 fluorescence spectrophotometer with 10 nm slit widths. Emission spectra were obtained at an excitation wavelength of 480 nm. Excitation spectra used emission at 600 nm.

RESULTS

Probe and assay design

The use of fluorescent tags in the nick translation PCR reaction is illustrated in Figure 1. As described by Holland *et al.* (2), the probe anneals to a region of one strand of PCR product downstream from one PCR primer. The 3' end of the probe is blocked from extension with a 3' phosphate. The probe is included with the other PCR reagents, and is degraded by the 5'−3' exonuclease activity inherent in *Taq* polymerase as primer is extended in every cycle. The probe is doubly labeled: a

Table 1. Primers and probes

Oligonucleotide	Sequence (5'−3')	Predicted Tm (°C)*	Strand Sense and Location in CFTR cDNA Sequence†
PCR pr1	CTTCACTTCTAATGATGATTATGGG	67	+ 1526−1550
PCR pr2	TGGCATGCTTTGAGACG	67	− 1706−1689
6FAM*wt*	6FAM TMR CATCTTTGGTGTTTCCTATGATGAATATp	70	+ 1650−1677
TET*mut*	TET TMR TATCATTGGTGTTTCCTATGATGAATATp	69	+ 1647−1677

The deletion region in the *wt* probe is underlined, the deletion site in the *mut* probe is indicated with an arrowhead. p = 3'-phosphate
* in PCR buffer with 4.2 mM free Mg^{2+} at 0.2 μM probe concentration
†Riordan,J.R. *et al.* (1989) *Science* 245, 1066−1073.

fluorescent indicator dye is at the 5′ end, and a quencher dye is attached internally via LAN. The linker arm nucleotide is a thymidine analogue which previously has been used in oligonucleotide energy transfer experiments (4). The proximity of indicator and quencher dyes in the intact probes reduces indicator dye fluorescence. Probe digestion during PCR restores indicator dye fluorescence if and only if nucleolytic cleavage occurs between the two dyes.

The degradation of two probes of different sequence can be monitored simultaneously if the two indicator dyes are spectrally distinguishable. This multiplex approach can be applied to the F508 and ΔF508 region by synthesizing one probe for each sequence, each labeled with a different indicator dye. Figure 2 illustrates a scheme for homogeneous allelic discrimination of F508 and ΔF508. Placing the deletion site between the two dyes permits discrimination between *wt* and *mut* target sequences because the polymerase will cleave only in duplex regions, and tends to displace several nucleotides at the duplex 5′ end before cleavage (2). Perfect-match probe-template duplexes experience probe cleavage several nucleotides to the 3′ side of the 5′ indicator dye, an event which increases indicator dye fluorescence. When the *wt* probe (6FAM*wt*) is bound to *mut* template or the *mut* probe (TET*mut*) is bound to *wt* template, enough mismatches occur in the region between the two dyes that the enzyme digests the duplex only between the quencher dye and the 3′ end of the probe. The latter cleavage does not enhance fluorescence because the resulting dye-labeled fragments still contain both dyes. Table 1 provides the sequences and melting properties of the PCR primers and the probes.

For the multiplex scheme to be effective, several criteria for the dyes must be met. First, the two indicator dyes must be spectrally distinct. Second, fluorescence quenching in the intact probe must be complete enough to detect increases in fluorescence at the concentrations of PCR probes and products which normally occur. Figure 3 shows the normalized fluorescence emission spectra of the individual dyes. The two indicator dyes, 6FAM and TET, have emission maxima which are separated by 20 nm. Greater separation of the emission spectra would also require greater separation of the excitation spectra, and thus would cause more difficulty exciting two dyes efficiently at a single wavelength.

TMR is a commonly used quencher and has been shown to provide effective energy transfer in oligonucleotides (5). In addition, TMR fluorescence can be used as an internal fluorescence reference to control for pipetting errors and evaporation during thermal cycling. The amount of quenching which occurs in the intact probes can be measured by comparing the fluorescence emission (and excitation) spectra of intact and cleaved probe. An analogous approach was used to measure quenching in labeled peptides (6).

Probe digestion

Degradation was most efficiently accomplished using ATP-dependent DNase; snake venom phosphodiesterase digested these oligonucleotides less completely (data not shown). After overnight reaction, the fluorescence spectra of 0.5 μM solutions of intact and degraded probes were measured. Figure 4 shows the excitation and emission spectra of the 6FAM*wt* probe and the TET*mut* probe. By comparing the emission intensities at the wavelength maxima of the digested and undigested indicator dyes, the quenching efficiency was calculated. This analysis showed that indicator dye quenching was 93% in 6FAM*wt* probe and 92% in TET*mut* probe.

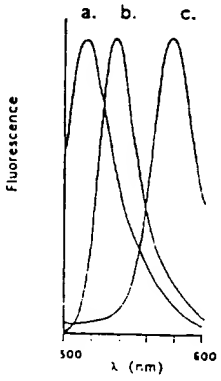

Figure 3. Normalized fluorescence emission spectra of separate dyes. Spectra were obtained in 20 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$ and 80 mM Tris–HCl at pH 8.9 using singly labeled oligonucleotide at 0.5 μM. a. 6FAM. b. TET. c. TMR.

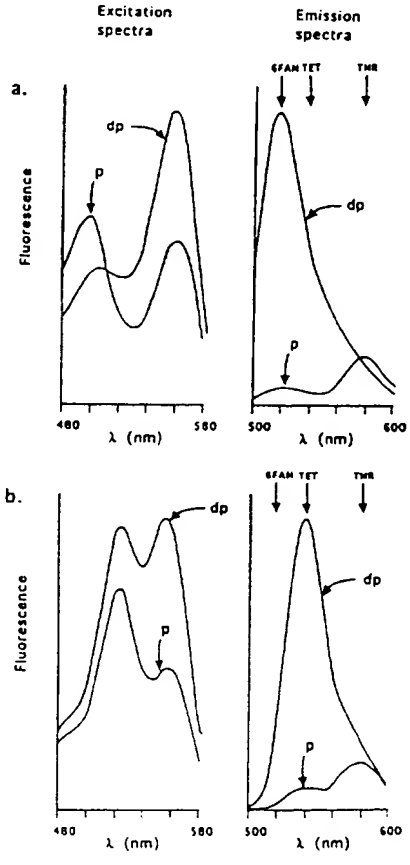

Figure 4. Excitation and emission spectra of intact (p) and digested (dp) probes. Digestion was accomplished with ATP-dependent DNase. a. 6FAM*wt* probe. b. TET*mut* probe.

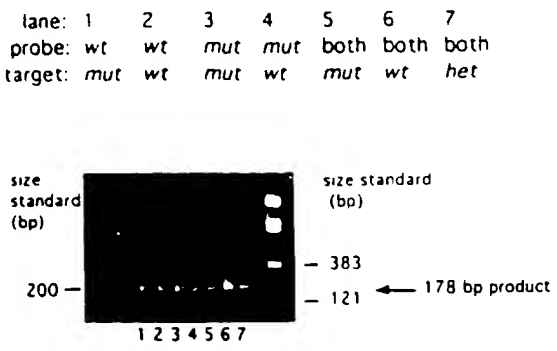

Figure 5. Agarose gel electrophoresis of PCR products. Lanes 1 and 2 contain 6FAM*wt* probe; lanes 3 and 4 contain TET*mut* probe; lanes 5–7 contain both 6FAM*wt* and TET*mut* probes. Lanes 1, 3 and 5 contain *mut* DNA; lanes 2, 4 and 6 contain *wt* DNA; and lane 7 contains *het* DNA.

PCR

Amplifications containing one or both probes (6FAM*wt* and TET*mut*) were performed as described above. Three types of genomic DNA were used: ΔF508 homozygote (*mut* DNA), heterozygote (*het* DNA), and F508 homozygote (*wt* DNA). The reactions were analyzed by three methods: agarose gel electrophoresis (5 μL), acrylamide gel electrophoresis on an automated sequencer (0.3 μL), and fluorescence emission of the PCR solution (50 μL).

Agarose gel electrophoresis. Figure 5 shows the results of agarose gel electrophoresis of seven separate PCR's containing various combinations of probe and target DNA. The specifically amplified 178 bp product shows similar band intensities for all probe combinations (and for amplifications without probe—data not shown), indicating that probe annealing to PCR template does not affect amplification efficiency. This result agrees with the observations of Holland *et al.*

Acrylamide gel electrophoresis. Figure 6 shows the automated sequencer results both as a gel display and as electropherogram profiles of three of the lanes. The gel was examined using bandpass filters at 531, 545, 560 and 580 nm, which correspond in the displays to blue, green, yellow and red, respectively. 6FAM maximally emits in the blue filter; TET appears green; and TMR appears red. The shapes of the emission spectra cause 6FAM to emit more in the green filter window than TET emits in the blue window. The electropherograms show multicomponented data which is not scaled to compensate for dye brightness. TET is approximately twice as bright as 6FAM on the automated sequencer.

The results show that indicator dye release is sequence-specific. Lane 8 contains the two probes in a non-cycled reaction. Lane 1 contains a reaction with 6FAM*wt* probe and *mut* DNA. Very little 6FAM (blue) is released during the PCR, although new, red bands appear, suggesting cleavage of the probe between TMR and the 3' end. Lane 2 contains a reaction with 6FAM*wt* probe and *wt* DNA. A new, blue band appears, indicating cleavage of the probe between the 6FAM and TMR. Lane 3 contains a reaction with TET*mut* probe and *mut* DNA. A new green band appears, again indicating cleavage between TET and TMR. Lane 4 contains a reaction with TET*mut* probe and *wt* DNA. Only new red bands appear, again indicating cleavage downstream of TMR. The red cleavage products apparently have anomalous mobility properties, as they must be longer products but have greater mobility than the singly-labeled cleavage products. Lanes 5 and 6 and 7 all contain both 6FAM*wt* probe and TET*mut* probe. Lane 5 contains a reaction with *mut* DNA (new green band); lane 6 contains a reaction with *wt* DNA (new blue band); and lane 7 contains a reaction with *het* DNA (new blue and green bands under the red). The results from the *het* DNA are difficult to view in the gel display because of the red background, but are clearly visible in the electropherograms. The electropherograms also show a small amount of nonspecific, high mobility cleavage product.

Solution-phase fluorescence. Figure 7 shows the fluorescence emission spectra of the three PCR's which contained both probes and the three types of DNA, and of the non-cycled reaction mixture (corresponding to lanes 5–7 of Figure 5 and lanes 5–8 of Figure 6). The two homozygotes show the λ_{max} values expected for the corresponding probe indicator dyes, whereas the heterozygote shows the broader indicator dye peak expected from a dye mixture. Measuring the ratios of the fluorescence intensities at 520, 535, and 580 nm permits evaluation of both the extent of cleavage (and therefore the nick translation PCR efficiency) and the type of target DNA.

Analysis of the fluorescence emission spectra requires normalization to the TMR emission peak in the initial probes' fluorescence, subtraction of the initial probes' fluorescence, and multicomponent analysis using overdetermined classical least squares to separate the contributions of the released indicator dyes. The spectral data were examined in 5 nm increments from 505 nm to 560 nm. The spectra of the individual indicator dyes were obtained from Figure 3 and the spectra of the initial probes' fluorescence from Figure 7 (spectrum 8). The resulting concentration ratios for released indicator dye in each experiment are given in Table 2.

The top panel provides the relative dye concentrations after multicomponent analysis and is corrected for the 20% increase in fluorescence emission intensity of 6FAM compared to TET when excited at 480 nm. The only simple explanation for the emission intensity difference between TET*mut* probe on *mut* target and 6FAM*wt* probe on *wt* target is that *Taq* polymerase digests these two perfect-match duplexes with a 2.6-fold (=6.2/2.4) ratio of efficiencies. The difference in degradation efficiency is assumed to be a function of the probe design and to be reproducible. Therefore the TET values are each increased by a factor of 2.6 to compensate for the efficiency difference to provide the numbers in the bottom panel. The multiplier is justifiable because the automated sequencer data showed that cleavage is almost completely specific. The percentages of each color are also given in the bottom panel. (The multiplier also assumes that the nonspecific cleavage of TET*mut* probe is also 2.6-fold less efficient than specific and nonspecific cleavage of 6FAM*wt* probe, an assumption which almost certainly is incorrect. The percentages of each color should represent a worst-case example.) As an experimental uncertainty of 20% or less should give satisfactory genomic analysis, the results show that the homogeneous detection can identify accurately the target DNA genotype.

lane:	1	2	3	4	5	6	7	8
probe:	*wt*	*wt*	*mut*	*mut*	both	both	both	both
target:	*mut*	*wt*	*mut*	*wt*	*mut*	*wt*	*het*	none

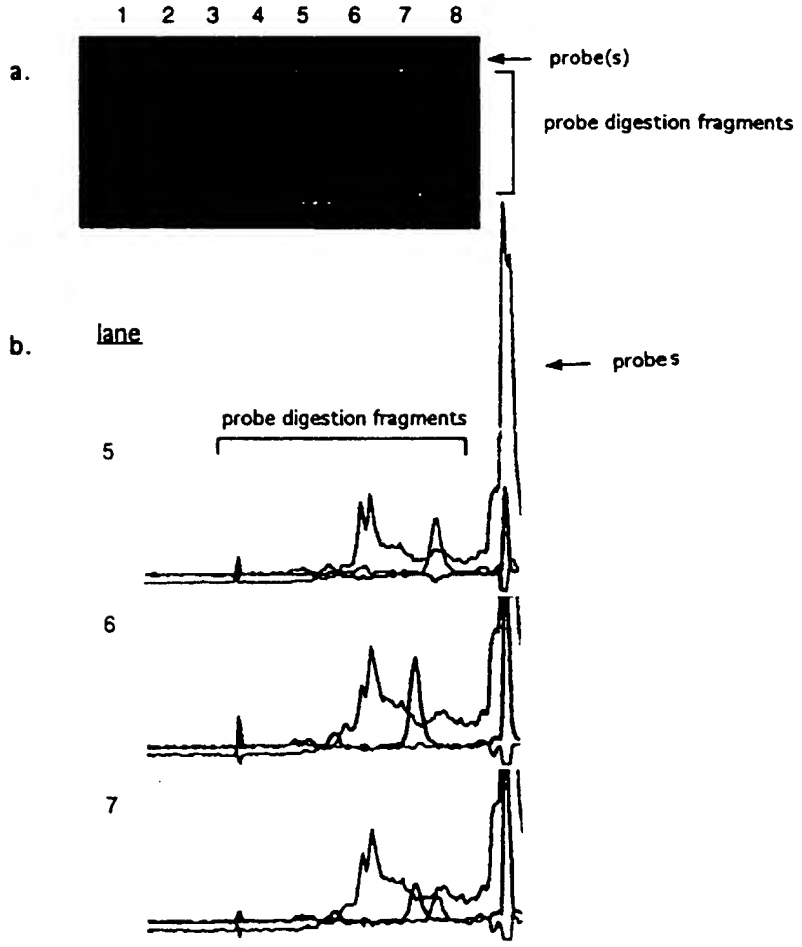

Figure 6. Size and color analysis of probes and nick-translation probe degradation products. Lanes 1–7 contain the same PCR reactions described in Figure. 5. Lane 8 contains a non-cycled reaction mixture with both 6FAM*wt* and TET*mut* probes. a. Gel display. b. Electropherograms of lanes 5, 6, and 7 after multicomponent analysis. No correction for dye intensities was applied.

Table 2. Relative amounts of 6FAM and TET in PCR solutions from analysis of spectra in Figure 7

	PCR solutions (corresponding to lanes 5–7 of Figures 5 and 6)		
	mut DNA (lane 5)	*wt* DNA (lane 6)	*het* DNA (lane 7)
(a) 6FAM:	0.52	6.2	3.2
TET:	2.4	0.45	1.5
(b) 6FAM:	0.52 (8%)	6.2 (84%)	3.2 (45%)
TET	6.2 (92%)	1.2 (16%)	3.9 (55%)

(a) Relative quantities of dyes after multicomponent analysis and correction for fluorescence efficiency. (b) Relative quantities of dyes after correction for cleavage efficiency.

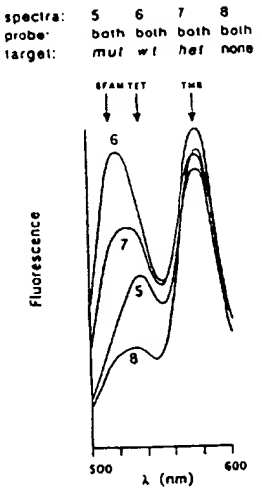

Figure 7. Uncorrected fluorescence emission spectra of the PCR products from lanes 5, 6, 7 and 8 described in Figures 5 and 6.

DISCUSSION

Table 2 shows where improvements in the method should be sought. The cleavage efficiencies of the two probes should be equalized (e.g., by probe and primer design, or change in PCR conditions) in order to reduce the value of the TET multiplier. The source of the high-mobility nonspecific peaks in Figure 6 should be identified and eliminated. The classical least squares analysis used for the multicomponent analysis could be changed to emphasize the data points at the peaks of the emission spectra. The indicator dyes could be improved to maximize spectral separation, and the excitation wavelength could be chosen so both dyes are equally excited. In addition, the effect of energy transfer in the intact probes (7) was ignored in the normalization and could be included in an iterative process. Following optimization of the method, statistical data comparing solution-phase fluorescence detection and gel-based separation methods should be obtained.

The nick-translation PCR method has disadvantages. First, it lacks the PCR product size information which is normally obtained by an accompanying separation method (e.g., gel electrophoresis, HPLC). Second, the multiplex capabilities are limited by the number of dyes required per locus (three dyes; one quencher and two indicators). Future efforts will include developing dye systems at new wavelengths in order to improve the multiplex potential.

Homogeneous, probe-based detection methods which rely on fluorescence energy transfer have been previously described (5, 8). These methods, however, require a post-PCR probing step. The duplex structures of Figure 2 show that the nick-translation method has an additional level of specificity compared to the probe-based assays. The specificity is due to the ability of *Taq* DNA polymerase to distinguish between complementary and uncomplementary nucleotides of the hybridized probe, and to cleave only at complementary nucleotides. The allelic discrimination arose solely from mismatch placement between the indicator and quencher dyes and not at all from the effect of base mismatch on probe annealing. The PCR anneal-extend temperature was 20°C below the predicted probe perfect-match T_m. A single-base-pair mismatch could not destabilize the probe-template duplex nearly enough to compensate for the lower temperature. Stringent probe-hybridization conditions were not necessary for successful allelic discrimination.

A homogeneous system has several obvious advantages relative to an analysis method requiring separation. Although collection of the data for Figure 7 and Table 2 required transfer of reaction mixtures from the PCR tube to a cuvette, this process is not intrinsic to the method. If fluorescence can be detected directly in the reaction vessel, false positives arising from worker or laboratory contamination with PCR products can be eliminated, because there is no reason to open the reaction tube post-PCR. The simplicity of the method renders it amenable to automated analysis, increasing analysis speed and reducing user error. The fluorescence measurement can be performed on a relatively low-cost fluorometer. The specificity of the probe-based system avoids error from PCR artifacts. The duplex structures of Figure 2 also suggest that discrimination with single-base-pair mismatches is possible with this type of assay.

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